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Effect of the addition of different catalase concentrations to a TRIS-egg yolk extender on quality and *in vitro* fertilization rate of frozen-thawed bull sperm

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submitted by

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ABSTRACT

The aim of this study was to investigate the effects of different concentrations of catalase in a TRIS-egg yolk extender on quality and embryonic development after *in vitro* fertilization of frozen-thawed bull sperm. For this purpose, from each of 7 bulls 2 ejaculates were collected and diluted with a TRIS-egg yolk extender containing 0, 5, 10, 15 or 20 IU catalase/mL. Sperm quality was evaluated 0, 3, 6, 12 and 24 h after thawing by using computer assisted analysis of motility and by flow cytometric assays. Embryonic development was determined after *in vitro* fertilization of bovine oocytes. Semen diluted with TRIS-egg yolk extender containing different concentrations of catalase showed more motile sperm, more sperm with intact plasma membranes, acrosomes and DNA, a lower amount of synthesis of reactive oxygen species and lower degree of lipid peroxidation of sperm compared to semen frozen without catalase ($P < 0.05$), but not before 3h after thawing. There was a dose-response relationship with the most prominent effect of 20 IU catalase/mL. However, the improvement of sperm quality had no effect ($P \geq 0.05$) on embryonic development after *in vitro* fertilization with 20 IU catalase/mL. In conclusion, the addition of catalase to the sperm extender improved sperm quality with no obvious effect on *in vitro* fertility.

Key words: bull sperm, cryopreservation, catalase, sperm quality, *in vitro* fertilization

Zusammenfassung

Ziel dieser Studie war es, die Auswirkungen verschiedener Katalasekonzentrationen in einem TRIS-Eigelb-Verdünner auf die Qualität kryokonservierter Bullenspermien und die Entwicklung von Embryonen *in vitro* zu untersuchen. Zu diesem Zweck wurden von 7 Bullen je 2 Ejakulate mit einem TRIS-Eigelb-Verdünner, der 0, 5, 10, 15 oder 20 IU Katalase/ml enthielt, kryokonserviert . Die Qualität der Spermien wurde 0, 3, 6, 12 und 24 h nach dem Auftauen mittels computergestützter Analyse der Spermienmotilität und verschiedener durchflusszytometrischer Assays bestimmt. Zusätzlich wurde die Embryonalentwicklung nach *in vitro*-Fertilisation von Rindereizellen untersucht. Alle verwendeten Katalasekonzentrationen hatten positive Auswirkungen auf die Motilität, die Integrität der Plasmamembran, des Akrosoms und der DNA sowie den Metabolismus der Spermien. Ferner reduzierte sich die Synthese reaktiver Sauerstoffspezies und der Grad der Lipidperoxidation der Spermien ($P<0.05$). Die Effekte auf die Spermaqualität nahmen mit steigender Katalasekonzentration zu, aber erst ab 3 Stunden nach dem Auftauen. Die Katalasekonzentration von 20 IU/ml hatte den stärksten Effekt auf die untersuchten Spermaparameter, führte aber nicht zu einer Erhöhung der Embryoentwicklungsrate nach *in vitro* Fertilisation. Zusammenfassend kann festgestellt werden, dass Katalase im TRIS-Eigelb-Verdünner die Qualität kryokonservierter Bullenspermien verbessert ohne jedoch die Fertilität *in vitro* zu erhöhen.

Schlüsselwörter: Bulle Sperma, Kryokonservierung, Katalase, Spermaqualität, *in-vitro* Fertilisation

Abbreviations

AM	Acetoxymethyl
AO	Acridine Orange
BP	Bodipy
BP M_{pos}	Sperm with mean LPO in high mitochondrial membrane potential
Ca^{2+}	Calcium
CASA	Computer Assisted Sperm Analysis
Cat	Catalase
COCs	Cumulus-Oocyte-Complexes
C_{pos}	Sperm with a high Intracellular Esterase Activity – Viable sperm
$C_{pos}M_{pos}$	Viable sperm with a high mitochondrial membrane potential
$C_{pos}M_{pos}F_{neg}PI_{neg}$	Viable sperm with a high mitochondrial membrane potential, an intact plasma membrane and a low calcium level
$C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$	Sperm with a high intracellular esterase activity, a high mitochondrial membrane potential, a low intracellular calcium level and intact plasma- and acrosome membrane
%DFI	Sperm with a high DNA Fragmentation Index
F_{neg}	Sperm with a low Intracellular Calcium Level
FI	Fluorescence Intensity
FSC-A	Forward Scatter Area
FSC-H	Forward Scatter Height
h	Hour / Hours
H_2O_2	Hydrogen peroxide
HE	Hydroethidine
HE M_{POS}	Hydroethidine fluorescence intensity in sperm with a high mitochondrial membrane potential
HMMP	High Mitochondrial Membrane Potential
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LPO	Lipid Peroxidation
M_{pos}	Sperm with a high Mitochondrial Membrane Potential

na	Term not included in the structure of final model
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric Oxide
O_2	Oxygen
O_2^-	Superoxide Anion
PE	Phycoerythrin
PI	Propidium Iodide
PI_{neg}	Sperm with an intact Plasma Membrane
PMAI	Sperm with an intact Plasma Membrane and Acrosome
PNA	Peanut Agglutinin Lectin
PNA_{neg}	Sperm with an intact acrosome
RMS	Rapid Motile Sperm
ROS	Reactive Oxygen Species
SSC-A	Side Scatter Area
SSC-H	Side Scatter Height
SQA	Sperm Quality Analyzer
TNE	Tris-NaCl-EDTA
VAP	Average Path Velocity

1. INTRODUCTION

Semen cryopreservation is widely used for reproductive purposes for more than a half century [27, 55]. Cryopreservation aims to interrupt the metabolic activities of sperm for a prolonged maintenance [11, 65]. Despite advances in semen cryopreservation during the last decades, still a diversity of damages occurs at molecular and cellular levels throughout the process of cryopreservation, which impairs function and fertilization potential of sperm [15, 32]. It is well-known that physical and chemical stresses are generated on spermatozoa during processes of freezing and thawing due to induction of osmotic stress, changes in temperature and generation of ice crystals [5, 17, 21, 27, 36]. The concentrations of reactive oxygen species (ROS) increase throughout freezing and thawing cycles, while the levels of antioxidants decrease [25, 29, 35].

Sperm produce physiologically low amounts of ROS such as superoxide anion (O_2^-), nitric oxide (NO) and hydrogen peroxide (H_2O_2) [7], which enhance their fertilization potential by increasing motility and inducing hyperactivation and capacitation [6, 42, 59]. Under physiological conditions, there is a balance between ROS production and antioxidant activity in the male reproductive system [56]. However, after cryopreservation this balance is disrupted in sperm due to an excessive ROS production and a decreased enzyme activity, which cause oxidative stress [1, 12]. Oxidative stress induces declines in viability [13, 14], motility and mitochondrial membrane potential of sperm, rises in lipid peroxidation (LPO) [8, 26, 34, 39, 40] and DNA damage [26, 41, 54] and disturbances of sperm-oocyte fusion [7] and embryo development after fertilization [3, 6]. To avoid oxidative stress during the freezing and thawing process, it is recommended to supplement antioxidants to the semen extender [22, 46]. The enzyme catalase is an antioxidant, which is physiologically present in the sperm cytoplasm and seminal plasma for the protection of sperm against ROS damage [47, 55]. As bovine semen contains only low amounts of catalase, oxidative stress could be lowered by adding catalase to the extender [9, 31]. Catalase catalyses the conversion from H_2O_2 to H_2O and oxygen (O_2) [2, 38], and inhibits thereby the chain reactions that lead to LPO. Furthermore, catalase removes O_2^-, which is generated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [13, 27, 30]. Thus, it prevents sperm damage caused by oxidative stress during the freezing and thawing cycles [16, 27]. The addition of different doses of catalase has positive effects on sperm function and fertilization rate, which are an improved sperm motility on bovine sperm (addition of 200 IU catalase/mL after cryopreservation) [30], a higher percentage of viable sperm on bovine sperm (addition of 100 IU catalase/mL after cooling at 5°C for 48 h (hours)) [27], an increase in acrosome and DNA integrity on bovine sperm (addition of 200 IU catalase/mL after cryopreservation) [30], a lower percentage of abnormal sperm and lower malondialdehyde concentrations in frozen-thawed sperm of mithun (Bos frontalis) bulls (addition of 50 IU catalase/mL before cooling) [51] besides an increased rate of cleaved zygotes and developing blastocysts as well as a decreased ROS generation on frozen-thawed sperm of boars (addition of 200 and 400 IU catalase/mL before cryopreservation) [55]. In the different studies mentioned above a high range of catalase concentrations was used, but up to now there is no information available regarding to the most effective dose of catalase addition before cryopreservation on sperm characteristics. For this reason, in the present study, we compared the impact of the addition of different concentrations of catalase to a TRIS-egg yolk extender on different sperm parameters and in addition we investigated the effect of the catalase concentration with the most positive impact on sperm quality on embryonic development rate after *in vitro* fertilization.

2. MATERIAL AND METHODS

2.1 Sperm Analysis

2.1.1 Chemicals and reagents

Chemicals and reagents used for the preparation of Tyrode's solution, Tris-NaCl-EDTA (TNE) buffer (1 mM EDTA, 0.01 M Tris, 0.15 M NaCl, pH 7.4), acridine orange (AO) staining buffer (1 mM EDTA, 0.1 M citric acid, 0.2 M Na_2HPO_4, 0.15 M NaCl, pH 6.0), acid detergent solution (0.08 N HCl, 15 M NaCl, 0.1% Triton-X 1000, pH 1.2) besides propidium iodide (PI) and Fluo-4 Acetoxymethyl (AM) were purchased from Sigma Aldrich Co. (Buchs, Switzerland) and peanut agglutinin lectin (PNA, Arachis hypogea) was ordered from GeneTex (Irvine, USA). The CellTrace™ Calcein Violet AM, 1,1', 3,3,3', 3'-Hexamethylindodicarbocyanine iodide [Mitoprobe™ $DilC_1$ (5)] and Hoechst 33342 from Thermo Fisher (Waltham, USA) as well as C11-BODIPY™ 581/591 (BP) and hydroethidine (HE) were purchased from Invitrogen (provided by Thermo Fisher, Waltham, USA).

2.1.2 Bulls

Ejaculates were obtained from seven healthy bulls (3 Brown Swiss, 2 Holstein Friesian, 1 Swiss Fleckvieh and 1 Original Braunvieh) from Swissgenetics (Zollikofen, Switzerland). The age of the bulls was 2.43 ±1.62 years (minimum: 1.2 years; maximum: 6.3 years). All bulls had a Non-Return Rate > 65% on day 56 after insemination.

2.1.3 Study Design

Two ejaculates from the bulls were collected twice a week (in total 14 ejaculates) with an interval of three to four days between collections. All ejaculates showed ≥ 70% progressive motile sperm. Ejaculates were divided into five aliquots, which were diluted with Triladyl® extender (Minitube GmbH, Tiefenbach, Germany) containing 0, 5, 10, 15 and 20 IU catalase/mL, respectively. All aliquots were cooled to 4°C in air, stored for 24 h in a refrigerator and afterwards frozen. After the cryopreservation process, all frozen sperm samples were thawed at 37°C for 30 seconds in a water bath and analysed immediately after thawing (0h) and after 3, 6, 12 and 24 h incubation at 37°C, respectively. The percentage of rapid motile sperm (RMS) was measured by using a computer assisted sperm analysis (CASA) system. The percentages of plasma membrane and acrosome intact (PMAI) sperm, sperm with a high intracellular esterase activity (C_{pos}), a high mitochondrial membrane potential (M_{pos}), a low intracellular (Ca^{2+}) level (F_{neg}) and an intact plasma membrane and acrosome (PNA_{neg}) ($C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$), sperm with a high DNA Fragmentation Index (%DFI), the mean fluorescence intensities of HE in M_{pos} sperm and BP in M_{pos} sperm (HE; total ROS, BP; LPO, M_{pos}: Mitoprobe™ $DilC_1$ (5); sperm with a high mitochondrial membrane potential) were measured by using flow cytometric assays.

2.1.4 Collection, evaluation, dilution and preservation of bull semen

Raw semen was collected by using an artificial vagina and progressive motility was estimated subjectively by using a phase contrast microscope with x100 magnification (Dilaux 20, Leitz, Wetzlar, Germany). Only ejaculates with ≥ 70% progressive motile sperm before freezing were used for the study. Sperm concentration quantified with a Sperm Quality Analyzer (SQA-Vb, SION AI. Company, Israel) ranged between 0.5 x 10^9 /mL and 2.0 x 10^9 /mL. Five aliquots of 8 mL Triladyl® extender (Minitube GmbH, Tiefenbach, Germany) were supplemented with 0, 5, 10, 15 and 20 IU catalase/mL, respectively, as described by Camara et al. (2011) [20]. Before the addition of semen, the extender was pre-warmed to 37°C. Semen was diluted in each aliquot to a final concentration of 60 x 10^6 sperm/mL.
All of the sperm samples were equilibrated at 4°C for 24 h and filled into 0.5 mL French straws (IMV Technologies, L'Aigle, France) at 4°C by using a fully automatic straw filling and sealing machine (MPP Quattro, Fa Minitube, Tiefenbach, Germany). Afterwards, all straws were put on the racks and frozen in horizontal position in a freezing chamber. Straws were frozen using a computer assisted freezing machine (Typ Digitcool 5300 3T, Fa. IMV L´Aigle, Frankreich) with a temperature decrease of 10°C/min to -140°C. After freezing, samples were immersed in liquid nitrogen (-196°C) and thereafter stored at least for 24 h at -196°C before analysis. After that, two straws were thawed by immersing them in a water bath (37°C for 30 s), opened and their content pooled, diluted to a concentration of 1.2 x 10^6 sperm/mL with pre-warmed (37°C) Tyrode's solution and kept at 37°C till analysis.

2.1.5 Computer assisted sperm analysis

Sperm motility was determined by using an IVOS II CASA system with the software version 14 (Hamilton Thorne Inc., Beverly, U.S.A.). Tyrode's solution and extended sperm samples were mixed equally to a final concentration of 30 x 10^6 /mL and analysed. The extended sperm samples with Tyrode's solution were stained by adding 80 µg/mL Hoechst 33342 and using the IVOS II system Ident Fluorescence Option ''Full Analysis'' in order to distinguish between non-sperm particles (other cells and extraneous particles in particular egg yolk components) and sperm [62]. Before motility measurements a 20 µm-deep semen analysis Leja 4-capillary chamber slide (Leja, Nieuw-Vennep, the Netherlands) was put on a preheated surface (37°C) and filled with semen. The objective calibration of the CASA machine was adjusted by Olympus 10X UV IVOS-II 160 mm. Also, the illumination of sperm was adjusted by using xenon lights. The kinematics of sperm were set up according to 40% progressive straightness and 10- μ m sperm cell travel distance. At least 1000 cells were examined in at least eight coincidentally chosen fields with 30 frames acquired per field at a frame rate of 60 Hz (the video frame capture speed for bull sperm) for each sample and the percentage of RMS defined by an Average Path Velocity (VAP) ≥ 50 µm/s was used for further analysis.

2.1.6 Flow cytometric analysis

Flow cytometric analyses were performed by using a CytoFlex® flow cytometer (Beckman Coulter, Fullerton, CA, USA). Three different lasers with wavelengths of 405 nm (80 mW laser output, violet laser), 488 nm (50 mW laser output, blue laser) and 638 nm (50 mW laser output, red laser) were used for analysis of semen samples. Fluorescence excitations were measured by using the following filters: V450 (450 ± 45 nm), FITC (525 ± 40 nm), PE (585 ± 42 nm), ECD (610 ± 20 nm), APC (660 ± 20 nm) and PC5.5 (690 ± 50 nm). Per semen sample, 10,000 sperm events were measured and saved as FCS files. The percentage of sperm with an intact plasma membrane and an intact acrosome, a low intracellular calcium (Ca^{2+})

level, a high intracellular esterase activity (C_{pos}) and a high mitochondrial membrane potential (HMMP) were determined by using a multicolour assay, which contained PI, phycoerythrin (PE) combined with PNA, CellTrace^{TM} Calcein Violet AM, Fluo-4 AM and Mitoprobe^{TM} $DilC_1$ (5). PI is a dye showing a red fluorescence after excitation with a 488 nm laser. It binds to the DNA of cells. As it is able to penetrate only cells with a defect plasma membrane, it is used to evaluate their plasma membrane integrity [33]. Sperm showing no red fluorescence after excitation with the 488 nm laser were defined as plasma membrane intact sperm (PI_{neg}). PNA conjugated with the orange fluorescent stain PE binds to the outer acrosome membrane of sperm with an acrosomal damage or an acrosome reaction [20, 23]. Sperm showing no orange fluorescence after excitation with the 488 nm laser were defined as sperm with an intact acrosome (PNA_{neg}). Sperm viability was measured by CellTrace^{TM} Calcein Violet AM, which is an indicator of intracellular esterase activity [52]. Viable and dead sperm cell populations were distinguished according to high (viable) and low (dead) violet fluorescence after excitation with a 405 nm laser. Fluo-4 AM binds to Ca^{2+} and fluoresces green after excitation with the 488 nm laser. Sperm with low intracellular Ca^{2+} levels (F_{neg}) (low green fluorescence) were separated from sperm with high intracellular Ca^{2+} levels (high green fluorescence) on a dot plot [28]. Mitochondrial membrane potential was measured by using Mitoprobe^{TM} $DilC_1$ (5). The fluorescent dye $DilC_1$ (5) diffuses into the cytosol of eukaryotic cells and accumulates in mitochondria. Mitochondria with a high membrane potential (M_{pos}) are producing a deep red fluorescence and those with a low mitochondrial membrane potential fluoresce red after excitation with a 638 nm laser light [21].

For the examination of each sperm sample with the multicolour assay, 5 µL of sperm were diluted in 239.75 µL of Tyrode's solution in a 250 µL reaction well of a 96-well-plate. Just prior to the performance of the assay, the fluorescent probes were combined in a master mix solution consisting of 0.375 µL Calcein Violet AM, 1.5 µL PI, 0.5 µL PNA, 2.5 µL Fluo-4 AM, and 0.375 µL $DilC_1$ (5) per reaction well. Thus, 5.25 µL of master mix were added to each reaction well. After 20 min of incubation at 37 °C sperm were analysed by flow cytometry.

For the evaluation of the results of the multicolour assay gates were set in the following order (Fig. 1): A) Sperm population was determined by using a dot plot with forward scatter area (FSC-A) on the x-axis and side scatter area (SSC-A) on the y-axis. B) Afterwards, doublet cells were discriminated from the sperm population by plotting FSC-A on the x-axis and forward scatter height (FSC-H) on the y-axis. C) Also, doublet cells were discriminated from the sperm population by plotting SSC-A on the x-axis and side scatter height (SSC-H) on the y-axis in order to ensure the count of single cells in the sperm population. D) The APC on the x-axis – V450 gate on the y-axis was used to define $C_{pos}M_{pos}$ sperm (viable sperm with a high mitochondrial membrane potential), E) a dot plot with FITC fluorescence on the x-axis and PC5.5 fluorescence on the y-axis to define $C_{pos}M_{pos}F_{neg}PI_{neg}$ sperm (viable sperm with a high mitochondrial membrane potential, an intact plasma membrane and a low Ca^{2+} level) F) a histogram with PE-A fluorescence on the x-axis to differentiate between PNA_{neg} and PNA_{pos} sperm and to define $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ sperm.

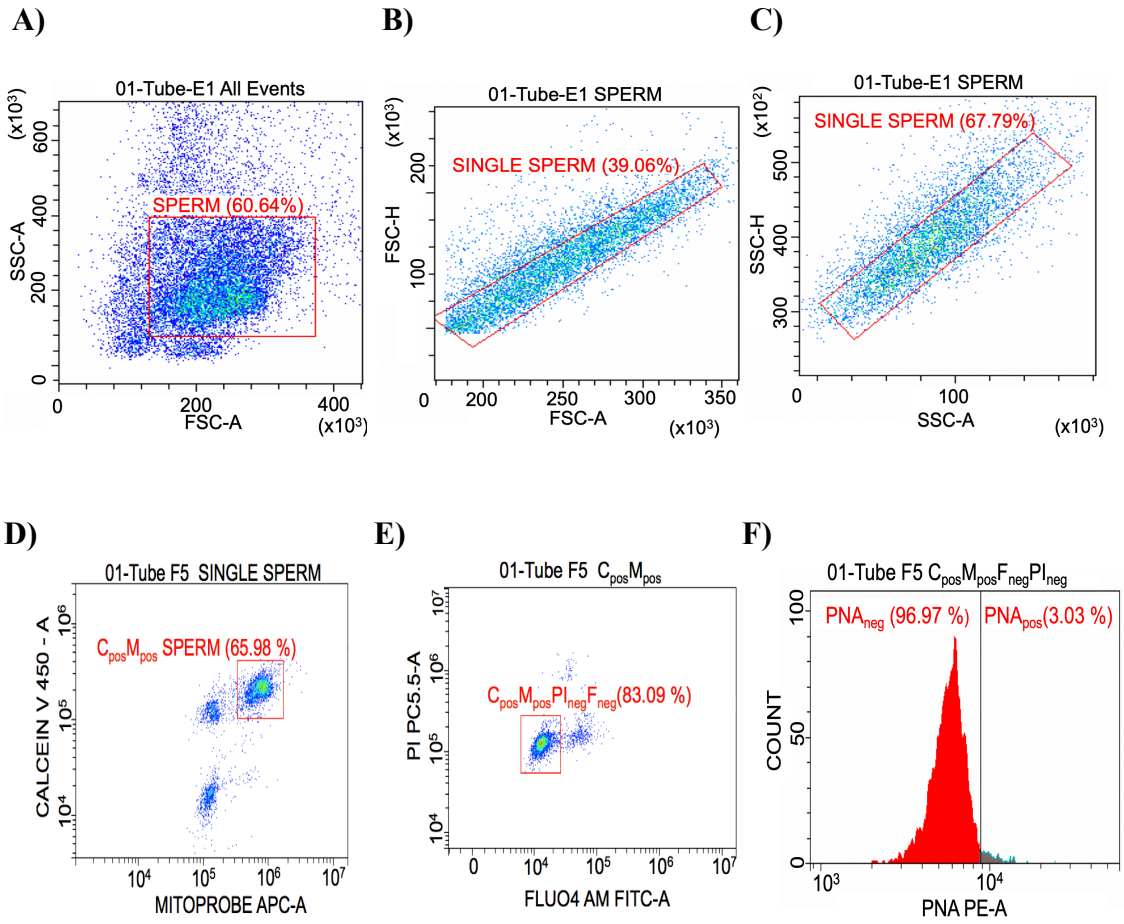

Figure 1: Order of gating sperm subpopulations with a high esterase activity (C_{pos}), a high mitochondrial membrane potential (M_{pos}), a low Ca^{2+} level (F_{neg}), an intact plasma membrane (PI_{neg}) and an intact acrosome: A) Sperm population gated by using FSC-A and SSC-A (SPERM); B) Population of single sperm determined by using FSC-A and FSC-H and C) SSC-A and SSC-H; D) Gating of the percentage of sperm with a high intracellular esterase activity (C_{pos}) and a high mitochondrial membrane potential (M_{pos}) determined by APC-A and Calcein V450-A fluorescence; E) Gating of sperm with a low Ca^{2+} level (F_{neg}) and an intact plasma membrane (PI_{neg}) sperm determined by FITC-A and PC5.5-A fluorescence in $C_{pos}M_{pos}$ sperm; F) Gating of sperm with an intact acrosome (PNA_{neg}) by using PE-A fluorescence of $C_{pos}M_{pos}F_{neg}PI_{neg}$ sperm. FSC-A (Forward Scatter - Area), FSC-H (Forward Scatter - Height), SSC-A (Side Scatter - Area), SSC-H (Side Scatter - Height).

For the evaluation of the PMAI sperm gates were set in the following order (Fig. 2): A) Sperm population was determined by using a dot plot with forward scatter area (FSC-A) on the x-axis and side scatter area (SSC-A) on the y-axis. B) Afterwards, doublet cells were discriminated from the sperm population by plotting FSC-A on the x-axis and forward scatter height (FSC-H) on the y-axis. C) Also, doublet cells were discriminated from the sperm population by plotting SSC-A on the x-axis and side scatter height (SSC-H) on the y-axis in order to ensure the count of single cells in the sperm population. D) The PE-A on the x- axis – PC5.5 gate on the y-axis was used to define PMAI sperm.

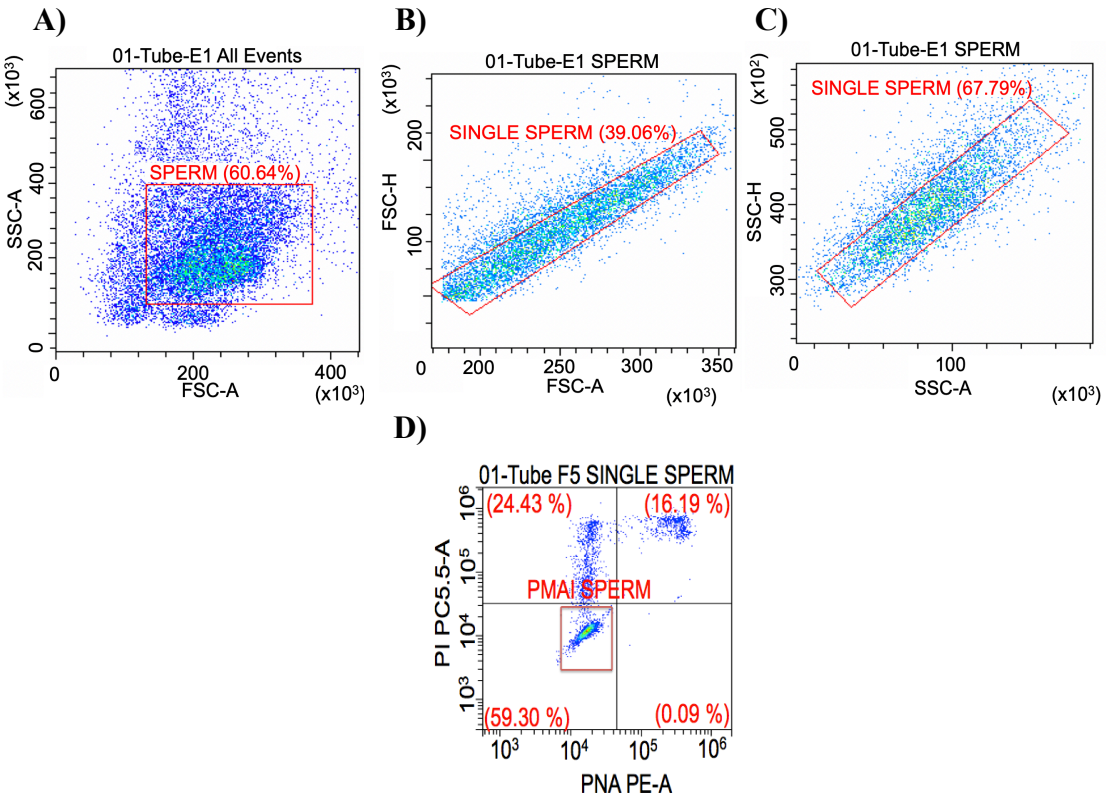

Figure 2: Order of gating sperm subpopulation with plasma membrane and acrosome intact sperm (PMAI); A) Sperm population gated by using FSC-A and SSC-A (SPERM); B) Population of single sperm determined by using FSC-A and FSC-H; C) SSC-A and SSC-H; D) Gating of the percentage of sperm with plasma membrane and acrosome intact sperm by using PE-A and PC5.5-A. FSC-A (Forward Scatter - Area), SSC-A (Side Scatter - Area), FSC-H (Forward Scatter - Height), SSC-H (Side Scatter - Height).

2.1.7 Lipid peroxidation and generation of reactive oxygen species

Mean fluorescence intensity of HE was used to evaluate intracellular ROS generation (superoxide anion, hydrogen peroxide, hydroxyl ion and peroxynitrite anion). The HE inserts within the DNA of the cell and exposes a red fluorescence in the cytosol of the nucleus after oxidation and excitation with a 488 nm blue laser [39]. The ROS was quantified by measuring the mean red fluorescence intensity of HE. By combing HE with Mitoprobe^{TM} DilC$_1$ the ROS generation of sperm with a HMMP was evaluated (5). LPO of the sperm plasma membrane was determined by using BP. This staining penetrates into the plasma membrane and shows a rise in green fluorescence after oxidation and excitation with a 488 nm blue laser. The LPO was quantified by measuring the mean green fluorescence intensity of BP. By combining BP with Mitoprobe^{TM} DilC$_1$ (5) LPO of sperm with a HMMP was evaluated (5).
For the simultaneous analysis of ROS and LPO in each sperm sample, 5 µL of sperm were diluted in 234.625 µL of Tyrode's solution in a 250 µL reaction well of a 96-well-plate. Just before the performance of the assay, the fluorescent probes were combined in a master mix solution consisting of 0.375 µL Mitoprobe^{TM} DilC$_1$ (5), 7.5 µl HE and 2.5 µl BP per reaction well. Thus, 10.375 µL of master mix were added to each reaction well and after 20 min of incubation at 37 °C, sperm were analysed by flow cytometry.
For the evaluation of ROS and LPO gates were set in the following order: A) Sperm population was determined by using a dot plot with forward scatter area (FSC-A) on the x-axis and side scatter area (SSC-A) on the y-axis. B) Afterwards, doublet cells were discriminated from the sperm population by plotting FSC-A on the x-axis and forward scatter height (FSC-H) on the y-axis. C) Also, doublet cells were discriminated from the sperm

population by plotting SSC-A on the x-axis and side scatter height (SSC-H) on the y-axis in order to ensure the count of single cells in the sperm population. D) The ECD-A on the x- axis and APC-A on the y-axis were used to quantify mean fluorescence of HE as an indicator for reactive oxygen species (ROS) in sperm with a high mitochondrial membrane potential (HE M_{pos}); E) The FITC-A on the x- axis and APC-A gate on the y-axis were used to quantify mean fluorescence FITC-A as an indicator for LPO in sperm with a high mitochondrial membrane potential (BP M_{pos}).

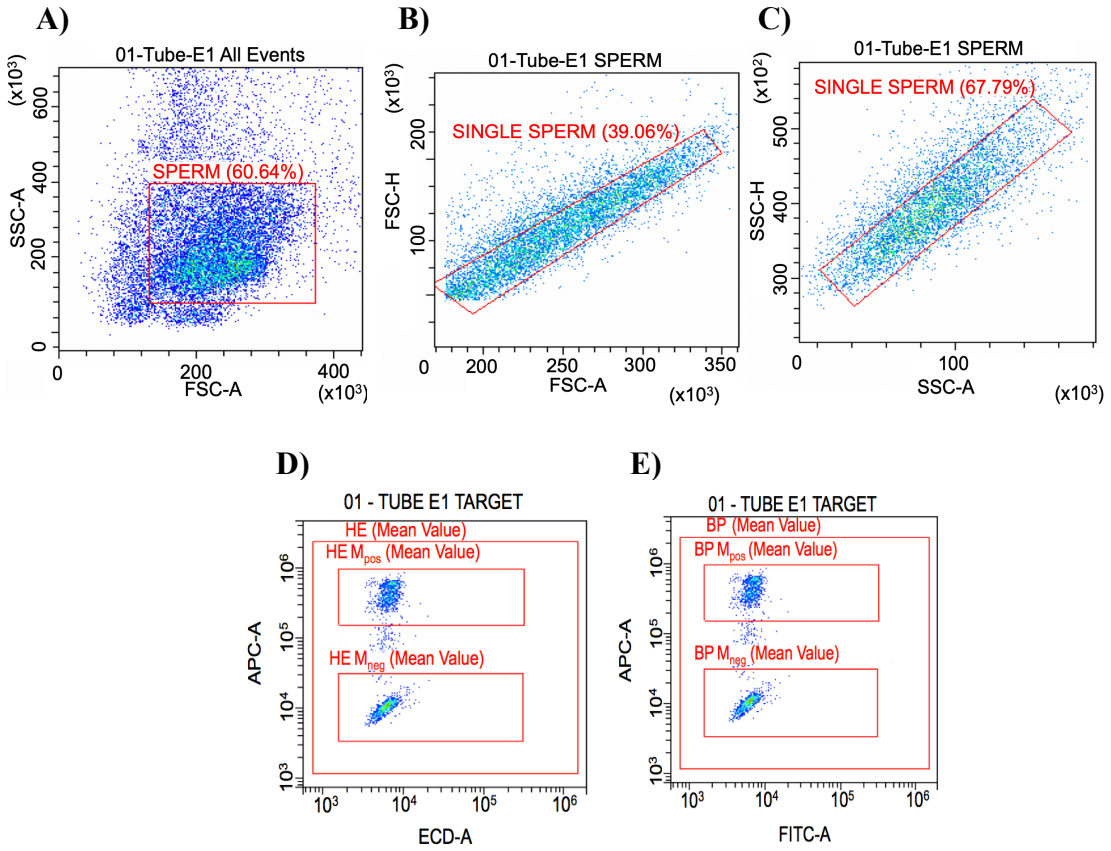

Figure 3: Order of gating to quantify reactive oxygen species (ROS) as the mean fluorescence intensity of hydroethidine (HE) in sperm with a high mitochondrial membrane potential (M_{pos}) and to evaluate the lipid peroxidation (LPO) as the mean fluorescence intensity of C11-BODIPY^{TM} 581/591 (BP) in sperm with a high mitochondrial membrane potential (M_{pos}): A) Sperm population gated by using FSC-A and SSC-A (SPERM); B) Population of single sperm determined by using FSC-A and FSC-H and C) SSC-A and SSC-H; D) Quantification of HE-fluorescence intensity after gating of sperm with a high mitochondrial membrane potential (HE M_{pos}); E) Quantification of BP-fluorescence intensity after gating of sperm with a high mitochondrial membrane potential (BP M_{pos}). FSC-A (Forward Scatter - Area), SSC-A (Side Scatter - Area), FSC-H (Forward Scatter - Height), SSC-H (Side Scatter - Height).

All samples were incubated for 30 minutes at 37 °C before flow cytometric analysis. The percentages of PMAI sperm, $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ sperm and the mean fluorescence intensities of BP M_{pos} and HE M_{pos} sperm (Figure 3) were analysed by using CytExpert 1.2 software (Beckman Coulter, Fullerton, CA, USA).

2.1.8 Sperm Chromatin Structure Assay

The Sperm Chromatin Structure Assay (SCSA™) was carried out as previously de-scribed [26]. Briefly, cryopreserved semen samples were diluted to 2 x 10^6 sperm/mL with TNE buffer (1 mM EDTA, 0.01 M Tris, 0.15 M NaCl, pH 7.4) after thawing of the straws. The

sperm suspension (200 µl) was mixed with 400 µl acid detergent solution (0.08 N HCl, 0.1% Triton-X 100, 0.15 M NaCl, pH 1.2) for 30 seconds and then stained with 1.2 mL AO (1 mM EDTA, 0.2 M Na_2HPO_4, 0.15 M NaCl, 0.1 M citric acid, pH 6.0) for a few seconds. After that, all samples were incubated for 3 minutes and investigated by using a Cytoflex® flow cytometer (Beckman Coulter, Fullerton, CA, USA). After excitation with 488 nm laser light acridine orange emits a green fluorescence (525 nm) if it is bound to double-stranded DNA and a red fluorescence (620 nm) if it is bound to single-stranded DNA (denatured) [28]. The DNA integrity was evaluated by determining the percentage of sperm with a high DFI (%DFI) using the software FCS Express (De Novo Software, Los Angeles, CA, USA).

2.2 *In vitro* production of bovine embryos

Bovine oocytes were collected from slaughterhouse ovaries that were transported at 38 °C in 0.9% NaCl from an abattoir in the vicinity of the laboratory. Follicles of 2 to 4 mm in diameter were aspirated using a 21 G needle connected to an aspiration pump and cumulus-oocyte-complexes (COCs) were collected under the stereomicroscope. All recovered COCs were washed twice in BO-Wash (IVF Bioscience, Denmark) and matured *in vitro* in groups of 10 in 50 µl micro droplets of BO-IVM medium (IVF Bioscience, Cornwall, United Kingdom) under mineral oil, at 38 °C, 5% CO_2 and saturated humidity. After 18-22 h of *in vitro* maturation (IVM), the COCs were transferred in groups of 20 to 200 µl droplets of BO-IVF medium (IVF Bioscience, Cornwall, United Kingdom). All COCs were randomly assigned to three different *in vitro* fertilization (IVF) groups: 1) Catalase-treated sperm: IVF with catalase-treated pooled sperm from 7 bulls; 2) Control sperm: IVF with non-treated pooled sperm from 7 bulls. All frozen sperm straws were thawed in a water bath at 37 °C for 30 s. In 1), 7 straws from catalase-treated sperm from 7 different bulls were thawed and pooled together. In 2), 7 straws from non-treated sperm from 7 different bulls were thawed and pooled together. In each IVF group, 0.25 mL of the thawed sperm were placed on top of 0.75 mL of Percoll 90% and centrifuged for 15 min at 600 x g. The pellet was resuspended in 0.75 mL of Hepes-buffered TALP medium and centrifuged for 3 min at 600 x g. Then, the pellet was resuspended in 100 µl of pre-equilibrated BO-IVF medium (IVF Bioscience, Cornwall, United Kingdom) and used to inseminate COCs with 2 x 10^6 sperm/mL. All COCs were co-incubated with sperm for 24 h at 38 °C, 5% CO_2 and saturated humidity (Day 0). 24 h after IVF, presumptive zygotes were denuded from the cumulus cells, placed in groups of 10 in 50 µl droplets of BO-IVC medium (IVF Bioscience, Cornwall, United Kingdom) covered with mineral oil and cultured at 38 °C, 5% CO_2, 6% O_2 and saturated humidity. 48 h after IVF, non-cleaved oocytes were discarded and cleaved embryos were cultured until Day 8. On Day 6, Day 7 and Day 8 of *in vitro* culture (IVC) embryos were observed under the stereomicroscope and the number of expanded and hatched blastocysts were registered for each group.

2.3 Statistical Analysis

Values (means and standard deviations) of sperm quality parameters were calculated in relation to incubation time and catalase treatment to define the dispersion and central tendency of the data. The changes of sperm parameters in the course of 24 h incubation were analysed by using growth curve analysis. The relation of the dependent variables RMS, PMAI, $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$, HE M_{pos}, BP M_{pos}, and %DFI to the increasing incubation time was presumed to be linear, quadratic or cubic; thereby, first-, second- and third-order orthogonal polynomials of incubation time were used to model the time curves. After defining the shape of the time curve, the fixed effect of *catalase treatment* (5 IU, 10 IU, 15 IU and 20 IU catalase/mL, respectively) was added in all time periods. The sperm parameter values of

control samples (catalase concentration 0 IU catalase/mL) were used as the baseline condition (control). Model parameters were estimated to define the effect of catalase treatment on the rate of change of sperm parameters over the course of 24 h incubation. Taking into account between- and within-bull variability, the ejaculate random effect (nested within bull) was included in the model structure; the intercept of the model was allowed to vary between bulls. The fixed effects of *catalase treatment* and *incubation time,* as well as their interaction term were stepwise included in the model structure. The subsequent progression model fit was evaluated exhibiting model comparisons based upon the − 2 log-likelihood ratio criterion (at 0.05 significance level). Normal approximations were used to estimate the parameter-specific P-values of the model. The *nlme* [53], *ggplot2* [66] and *lattice* [57] packages in R version 3.1.3 [63] were used to carry out statistical analysis, graphical illustration and processing of the data of the results. Least-squares means and linear contrasts for the factor combination incubation x catalase treatment were computed using the lsmeans statistical package for R [43]. The significance levels were adjusted using the Tukey method for comparing a family of five estimates. The differences in the developmental rates of embryos that were produced using catalase-treated vs. control sperm were assessed with the chi-square test for equality of proportions (two-sided), at a 0.05 significance level.

3. RESULTS

Descriptive statistics of sperm parameters in relation to incubation time and catalase treatment are presented in Table 1. The changes of characteristics of sperm treated with different catalase doses in the course of a 24 h incubation period are demonstrated in Figures 4 to 9. Growth curve analysis was performed in order to describe the effects of incubation time and catalase treatment (doses of 0, 5, 10, 15 and 20 IU catalase/mL) on sperm parameters. The fixed effects of *incubation time* and *catalase treatment* as well as their interaction term *incubation time* x *catalase treatment* on sperm parameters are presented in Table 2 (estimates of b coefficients ± SEM and P value). As expected, the assessed sperm parameters were affected by the increasing time of incubation ($P<0.05$ for the fixed effect of incubation time in all cases; Table 2). The changes of sperm parameters in the control group followed a cubic-shaped curve in the course of the 24 h incubation, with exception of PMAI sperm that exhibited a quadratic relation to incubation time as well as $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ sperm that decreased in a linear way over time (Table 2; Figures 4 to 9).

Table 1: Characteristics of cryopreserved bovine sperm diluted with extender without (Control) and with addition of different doses of catalase (Cat; 5 IU/mL, 10 IU/mL, 15 IU/mL and 20 IU/mL) at five different incubation time points (0h, 3h ,6h, 12h and 24h). Values are means ± SD of 2 ejaculates in each of 7 bulls (in total 14 ejaculates).

		Incubation Time				
Parameters	**Groups**	**0h**	**3h**	**6h**	**12h**	**24h**
RMS (%)	Control	44.66 ± 13.30	33.32 ± 21.91^{a}	18.02 ± 21.46^{a}	2.11 ± 6.06^{a}	0.02 ± 0.04
	Cat 5 IU/mL	45.49 ± 15.34	43.78 ± 14.67^{b}	39.45 ± 13.70^{b}	17.82 ± 12.58^{b}	0.07 ± 0.08
	Cat 10 IU/mL	44.60 ± 15.87	43.74 ± 14.18^{b}	41.11 ± 13.51^{b}	22.39 ± 13.50^{bc}	0.28 ± 0.43
	Cat 15 IU/mL	45.76 ± 14.50	43.66 ± 14.63^{b}	41.08 ± 13.82^{b}	25.57 ± 15.44^{c}	0.44 ± 0.67
	Cat 20 IU/mL	45.39 ± 15.69	42.66 ± 14.80^{b}	40.91 ± 15.04^{b}	25.11 ± 13.71^{c}	0.60 ± 0.68
PMAI (%)	Control	57.43 ± 10.98	55.68 ± 10.06	52.07 ± 10.67	38.16 ± 18.17^{a}	8.72 ± 7.12^{a}
	Cat 5 IU/mL	57.94 ± 11.62	56.07 ± 11.23	52.10 ± 10.30	47.05 ± 10.12^{b}	18.24 ± 9.82^{b}
	Cat 10 IU/mL	58.29 ± 11.21	56.00 ± 10.14	52.20 ± 10.02	47.60 ± 9.76^{b}	22.61 ± 8.29^{c}
	Cat 15 IU/mL	58.06 ± 11.16	56.41 ± 10.93	52.06 ± 10.44	47.95 ± 10.44^{b}	25.74 ± 10.59^{c}
	Cat 20 IU/mL	57.46 ± 11.29	55.09 ± 10.79	52.12 ± 10.90	47.58 ± 10.72^{b}	27.73 ± 11.08^{c}
$C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ (%)	Control	53.99 ± 11.02	52.17 ± 10.27	41.69 ± 18.54	20.75 ± 21.63^{a}	0.00 ± 0.00^{a}
	Cat 5 IU/mL	53.97 ± 11.50	52.60 ± 11.34	45.40 ± 11.40	40.05 ± 11.17^{b}	8.09 ± 8.79^{b}
	Cat 10 IU/mL	54.01 ± 11.18	51.73 ± 10.04	45.58 ± 11.07	42.68 ± 9.83^{b}	14.14 ± 8.60^{c}
	Cat 15 IU/mL	54.29 ± 11.68	52.32 ± 11.34	45.52 ±10.72	43.06 ± 10.26^{b}	17.68 ± 10.04^{c}
	Cat 20 IU/mL	53.35 ± 12.07	51.41 ± 11.49	45.50 ± 12.01	42.35 ± 11.05^{b}	20.08 ± 11.36^{c}
HE M_{pos} (FI x 100)	Control	105.32 ± 7.12	111.40 ± 8.82	121.40 ± 24.22	174.0 ± 123.0^{a}	454.0 ± 118.50^{a}
	Cat 5 IU/mL	104.80 ± 7.74	110.70 ± 7.17	108.30 ± 22.23	118.70 ± 7.36^{b}	172.30 ± 120.86^{b}
	Cat 10 IU/mL	106.48 ± 7.74	110.70 ± 8.07	114.10 ± 6.03	118.50 ± 7.90^{b}	131.50 ± 26.58^{c}
	Cat 15 IU/mL	106.0 ± 7.00	110.10 ± 7.34	114.40 ± 7.04	118.70 ± 9.98^{b}	126.70 ± 21.92^{bc}
	Cat 20 IU/mL	105.10 ± 7.16	108.80 ± 7.42	113.30 ± 6.28	115.20 ± 9.60^{b}	120.60 ± 19.87^{c}
BP M_{pos} (FI x 100)	Control	31.50 ± 11.59	21.60 ± 0.67	23.60 ± 2.52	43.0 ± 17.28	95.40 ± 42.32^{a}
	Cat 5 IU/mL	31.10 ± 11.50	20.90 ± 0.79	29.0 ± 28.57	26.0 ± 2.44	41.40 ± 17.62^{b}
	Cat 10 IU/mL	31.30 ± 12.22	20.90 ± 1.06	21.40 ± 0.90	25.0 ± 1.74	34.50 ± 5.12^{b}
	Cat15 IU/mL	31.0 ± 11.45	20.80 ± 0.92	21.40 ± 0.88	25.20 ± 1.87	32.90 ± 3.89^{b}
	Cat 20 IU/mL	31.0 ± 11.48	20.60 ± 0.85	21.20 ±0.71	24.80 ± 1.70	31.70 ± 3.56^{b}
%DFI (%)	Control	8.83 ± 8.13	10.68 ± 8.65	14.06 ± 8.67	52.00 ± 16.16^{a}	65.57 ± 12.48^{a}
	Cat 5 IU/mL	8.63 ± 8.46	9.68 ± 8.66	11.05 ± 9.30	15.01 ± 11.81^{b}	23.12 ± 14.96^{b}
	Cat 10 IU/mL	8.85 ± 7.69	9.56 ± 8.47	10.50 ± 8.64	13.84 ± 11.05^{b}	17.92 ± 12.43^{c}
	Cat 15 IU/mL	8.50 ± 7.25	9.29 ± 7.97	10.36 ± 8.35	12.77 ± 10.96^{b}	15.96 ± 12.12^{c}
	Cat 20 IU/mL	9.13 ± 8.05	9.42 ± 8.07	10.11 ± 8.46	12.61 ± 10.51^{b}	15.74 ± 11.80^{c}

RMS (Rapid Motile Sperm), PMAI (sperm with an intact plasma membrane and acrosome), $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ (sperm with a high intracellular esterase activity (C_{pos}), a high mitochondrial membrane potential (M_{pos}), a low intracellular calcium (Ca^{2+}) level (F_{neg}), an intact plasma membrane (PI_{neg}) and acrosome (PNA_{neg}), %DFI (sperm with a high DNA Fragmentation Index), HE M_{pos} (mean hydroethidine fluorescence of sperm with a high mitochondrial membrane potential), BP M_{pos} (mean C11-BODIPY^{TM} 581/591 fluorescence of sperm with a high mitochondrial membrane potential), Cat (catalase), FI (mean fluorescence intensity). Values with different letters within the same column and the same sperm parameter differ ($P<0.05$) for each sperm parameter.

Table 2: Estimated b coefficients (±SEM) and the respective P values for the fixed effect of incubation time (Incubation: linear, quadratic and cubic polynomial) and catalase treatment (Catalase: 0 IU/mL (Control), 5 IU/mL, 10 IU/mL, 15 IU/mL, 20 IU/mL) and their interaction term Incubation x Catalase on the values of six sperm parameters. The effect of the incubation time polynomial that the best described the shape of data in terms of goodness-of-fit of the model is indicated.

Fixed effect	RMS	PMAI	$C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$	%DFI	HE M_{pos} (FI x 100)	BP M_{pos} (FI x 100)
(Intercept)	20.11±3.90 (P<0.05)	42.39±3.29 (P<0.05)	34.50±3.49 (P<0.05)	30.22±3.37 (P<0.05)	189.0±6.70 (P<0.05)	44.20±5.20 (P<0.05)
Control linear term	-286.03±23.90 (P<0.05)	-337.07±12.72 (P <0.05)	-385.17±13.87 (P<0.05)	420.71±12.56 (P<0.05)	2280.0±101.0 (P<0.05)	243.0±23.0 (P<0.05)
Control quadratic term	146.21±20.74 (P<0.05)	-48.15±11.63 (P<0.05)	22.11±13.39 (P>0.05)	-58.18±12.48 (P<0.05)	na	na
Control cubic term	11.59±18.58 (P>0.05)	na	na	130.36±12.08 (P<0.05)	na	na
Condition: Cat 5 IU/mL	10.02±1.13 (P<0.05)	3.86±0.66 (P<0.05)	5.91±0.84 (P<0.05)	-16.71±0.74 (P<0.05)	-67.0±7.10 (P<0.05)	-7.0±1.40 (P<0.05)
Condition: Cat 10 IU/mL	11.14±1.32 (P<0.05)	4.91±0.79 (P<0.05)	7.54±0.97 (P<0.05)	-18.09±0.85 (P<0.05)	-73.0±7.20 (P<0.05)	-8.20±1.40 (P<0.05)
Condition: Cat 15 IU/mL	11.80±1.35 (P<0.05)	5.59±0.80 (P<0.05)	8.37±0.97 (P<0.05)	-18.86±0.86 (P<0.05)	-73.0±7.0 (P<0.05)	-7.40±1.30 (P<0.05)
Condition: Cat 20 IU/mL	11.53±1.21 (P<0.05)	5.49±0.71 (P<0.05)	8.24±0.88 (P<0.05)	-18.90±0.78 (P<0.05)	-74.0±7.10 (P<0.05)	-6.70±1.40 (P<0.05)
Linear term x Cat 5 IU/mL	-25.40±23.25 (P>0.05)	72.34±12.48 (P<0.05)	75.82±15.79 (P<0.05)	322.44±13.89 (P<0.05)	-1817±134.0 (P<0.05)	-242.0±25.80 (P<0.05)
Quadratic term x Cat 5 IU/mL	-132.80±21.05 (P<0.05)	-12.79±12.31 (P>0.05)	-83.76±15.65 (P<0.05)	65.43±13.91 (P<0.05)	na	na
Cubic term x Cat 5 IU/mL	51.27±20.01 (P<0.05)	na	na	126.26±13.80 (P<0.05)	na	na
Linear term x Cat 10 IU/mL	-15.95±26.95 (P>0.05)	101.31±15.18 (P<0.05)	121.43±18.27 (P<0.05)	-357.90±16.25 (P<0.05)	-2108.0±135.0 (P<0.05)	-273.0±27.30 (P<0.05)
Quadratic term x Cat 10IU/mL	-167.23±25.07 (P<0.05)	0.51±14.95 (P>0.05)	-79.72±18.12 (P<0.05)	56.49±16.36 (P<0.05)	na	na
Cubic term x Cat 10 IU/mL	38.71±24.13 (P>0.05)	na	na	124.85±16.25 (P<0.05)	na	na
Linear term x Cat 15 IU/mL	-16.11±28.24 (P>0.05)	123.10±16.10 (P<0.05)	144.47±18.86 (P<0.05)	369.55±16.80 (P<0.05)	-2053.0±133.0 (P<0.05)	-228.0±26.60 (P<0.05)
Quadratic term x Cat 15 IU/mL	-173.99±27.37 (P<0.05)	8.35±16.03 (P>0.05)	-69.52±18.90 (P<0.05)	53.37±17.24 (P<0.05)	na	na
Cubic term x Cat 15 IU/mL	24.12±26.60 (P>0.05)	na	na	130.38±17.20 (P<0.001)	na	na
Linear term x Cat 20 IU/mL	-9.97±27.96 (P>0.05)	142.72±15.90 (P<0.05)	168.91±18.40 (P<0.05)	-371.18±16.50 (P<0.001)	-2065.0±135.0 (P<0.05)	-218.0±27.0 (P<0.05)
Quadratic term x Cat 20 IU/mL	-177.81±27.94 (P<0.05)	12.59±16.29 (P>0.05)	-68.56±19.05 (P<0.05)	54.11±17.59 (P<0.05)	na	na
Cubic term x Cat 20 IU/mL	22.357±27.66 (P>0.05)	na	na	132.30±18.0 (P<0.05)	na	na

RMS (Rapid Motile Sperm), PMAI (sperm with an intact plasma membrane and acrosome), $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ (sperm with a high intracellular esterase activity (C_{pos}), a high mitochondrial membrane potential (M_{pos}), a low intracellular calcium (Ca^{2+}) level (F_{neg}), an intact plasma membrane (PI_{neg}) and acrosome (PNA_{neg}), %DFI (sperm with a high DNA Fragmentation Index), HE M_{pos} (mean fluorescence of hydroethidine of sperm with a high mitochondrial membrane potential), BP M_{pos} (mean fluorescence of C11-BODIPY^{TM} 581/591 of sperm with a high mitochondrial membrane potential), Cat (catalase), FI (mean fluorescence intensity), na: term not included in the structure of final model. The significant differences are indicated with a P<0.05.

As indicated by the sign of the b coefficients for the effect of catalase treatment on the overall values of sperm parameters (Table 2), catalase had a beneficial effect on the quality of sperm already at the concentration of 5 IU catalase/mL. Overall, catalase-treated sperm (5, 10, 15 and 20 IU catalase/mL) had higher RMS, PMAI and $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ values ($b>0$, $P<0.05$ for the effect of catalase treatment on overall sperm parameter values) besides lower %DFI ($b<0$, $P<0.05$ for the effect of catalase treatment on overall sperm parameter values) compared to the control group (Table 2). All catalase treatments resulted in lower values of fluorescence intensity of HE M_{pos} sperm and BP M_{pos} sperm in overall ($b>0$, $P<0.05$ for the effect of catalase treatment on overall HE M_{pos} sperm and BP M_{pos} sperm values; Table 2) compared to the control group.
The effect of catalase treatment (5, 10, 15 and 20 IU catalase/mL) on the rate of change of sperm parameters (linear term of incubation time) and the shape of the curve (quadratic and/or cubic term of incubation time) are presented in Table 2, in form of estimated b coefficients and the related P values. As shown in Figure 4, the rate of change of RMS was similar between catalase-treated sperm and control ($P>0.05$ for the effect of catalase treatment on the linear term of incubation time; Table 2), with all groups showing similar initial values at 0 h and reducing close to zero after 24 h incubation (Table 1). However, catalase-treated sperm showed a smoother decrease of RMS during the first 12 h of incubation (Figure 4). Because of this, RMS values of sperm cryopreserved with all different catalase concentrations were higher than those of the control group at 3h, 6h and 12h ($P<0.05$, Table 1). 12 h after thawing sperm frozen in extender with 15 IU and 20 IU catalase/mL showed higher RMS values than those frozen with an extender containing 5 IU catalase/mL concentrations ($P<0.05$, Table 1).

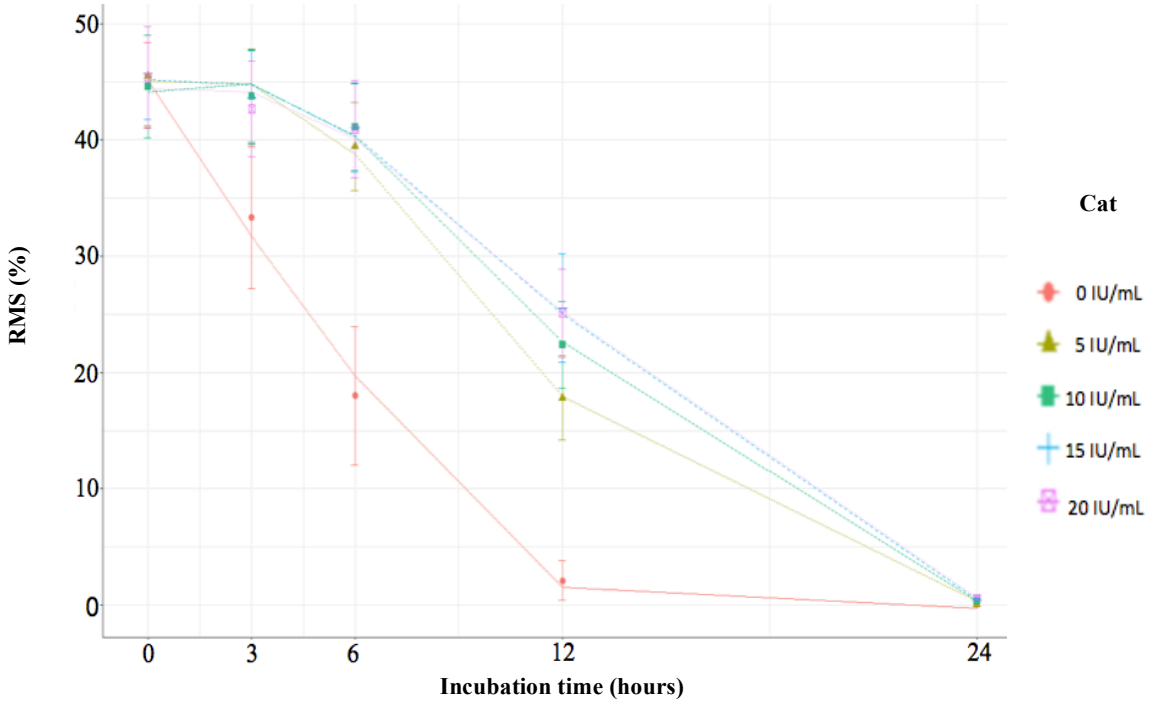

Figure 4: Mean values (±SEM) and model-predicted time curves of rapid motile sperm (RMS) in cryopreserved bovine sperm diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

As demonstrated in Figures 5 and 6, values of PMAI and $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ decreased in the control group in a quadratic and linear manner in the course of the 24 h incubation, respectively (Table 2). All concentrations of catalase weakened the rate of change of the above-mentioned parameters ($P<0.05$ for the effect of catalase treatment on the linear term of incubation at both parameters; Table 2), with catalase-treated sperm having higher PMAI and $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ values than the control after 12 and 24 h of incubation ($P<0.05$; Table 1). At 24 h of incubation sperm diluted with extenders containing 10 IU, 15 IU and 20 IU catalase/mL of showed higher PMAI and $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ values than those frozen with an extender containing 5 IU catalase/mL ($P<0.05$).

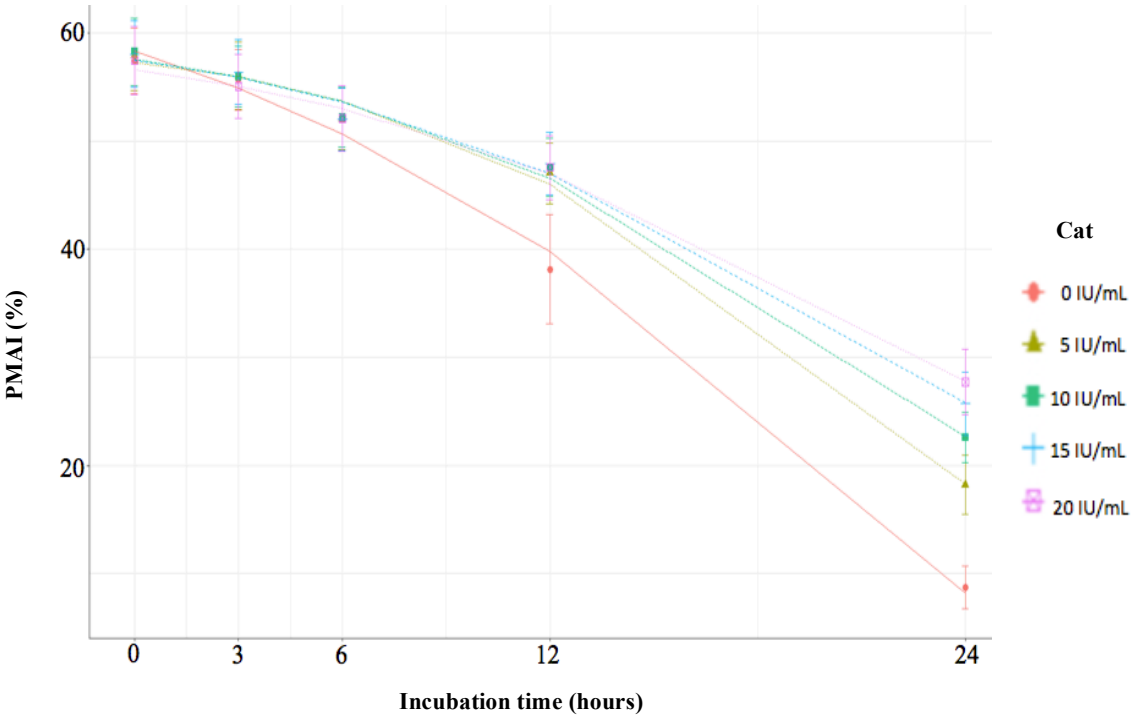

Figure 5: Mean values (±SEM) and model-predicted time curves of plasma membrane and acrosome intact (PMAI) cryopreserved bovine sperm diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

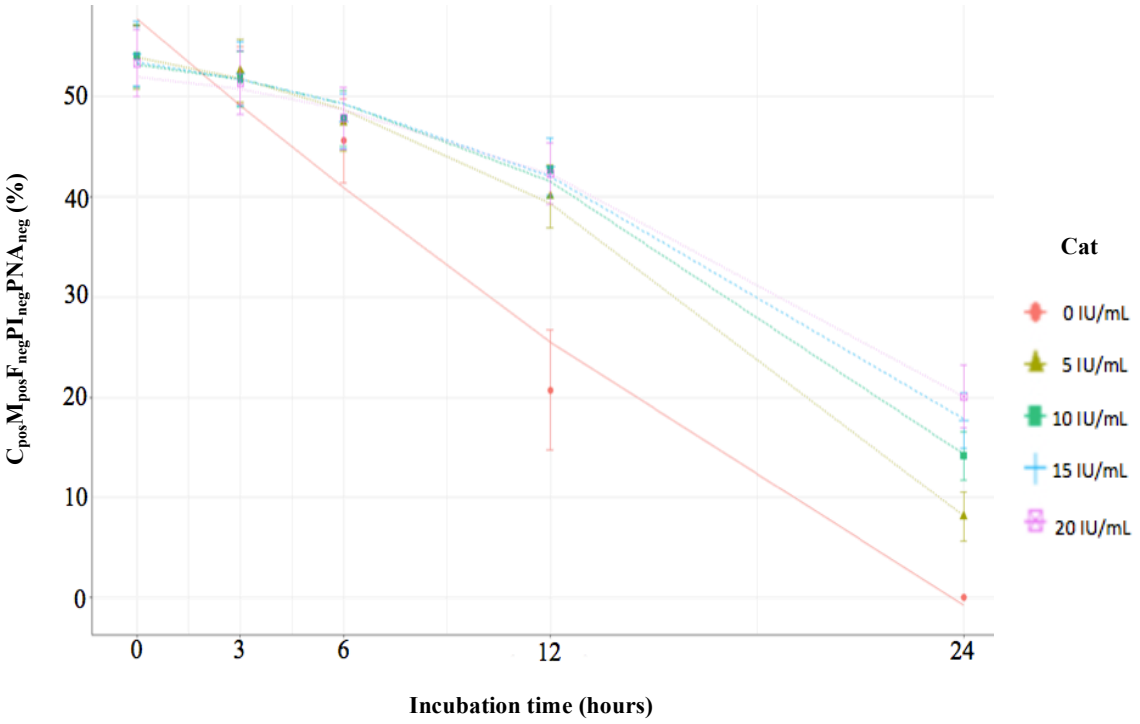

Figure 6: Mean values (±SEM) and model-predicted time curves of cryopreserved bovine sperm with a high intracellular esterase activity, a high mitochondrial membrane potential, a low intracellular calcium (Ca^{2+}) level and intact plasma membranes and acrosomes ($C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$) diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

The shape of the curve describing the best response of the HE fluorescence in M_{pos} sperm of the control group to increasing incubation time was the linear one ($P<0.05$ for the linear term of incubation; Table 2). The values for HE fluorescence in M_{pos} sperm showed a smoother rate of increase over 24 h ($P<0.05$ for the effect of catalase treatment on the linear (HE M_{pos}) term of incubation time; Table 2) as shown in Figure 7. Therefore, catalase-treated sperm (5, 10, 15 and 20 IU/mL) showed lower values for HE fluorescence in M_{pos} sperm after 12 and 24 h incubation ($P<0.05$ for the effect of catalase treatment on the linear term of incubation; Table 2) compared to the control group. At 24 h after thawing sperm cryopreserved with 10 IU and 20 IU catalase/mL showed a lower HE fluorescence in M_{pos} sperm than those frozen with 5 IU catalase/mL ($P<0.05$, Table 1).

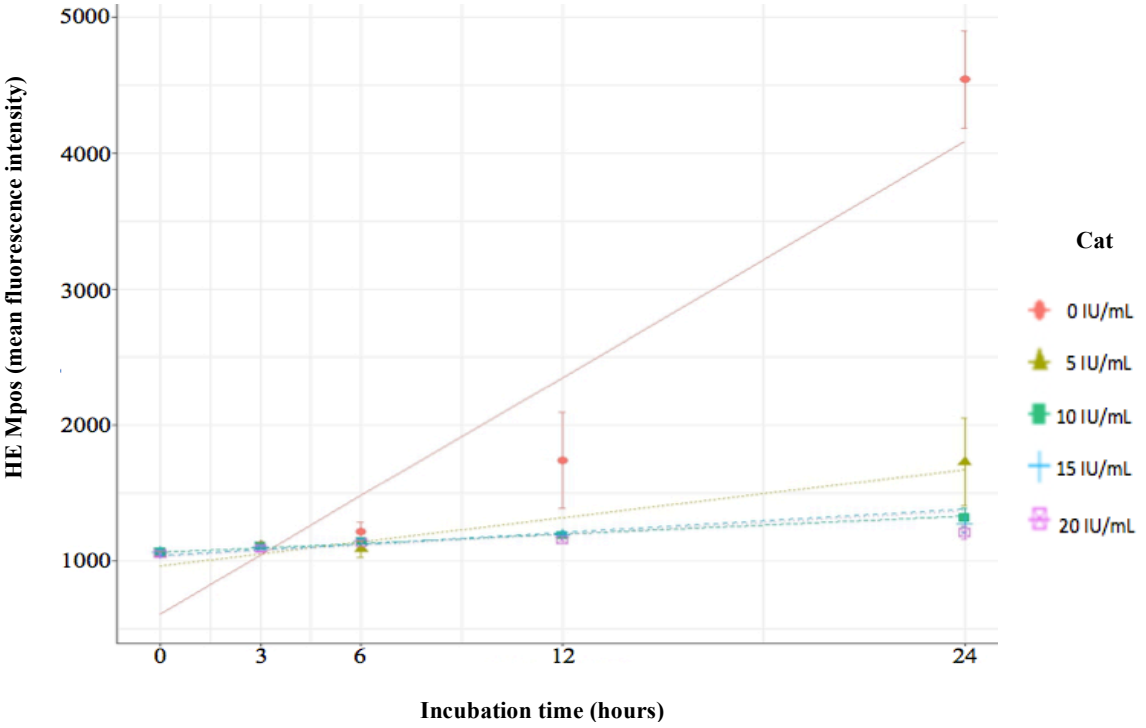

Figure 7: Mean values (±SEM) and model-predicted time curves of hydroethidine fluorescence in cryopreserved bovine sperm with a high mitochondrial membrane potential (HE M_{pos}) diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

As demonstrated in Figure 8, control samples indicated a linear increase of BP fluorescence in M_{pos} sperm ($P<0.05$ for the effect of catalase treatment on the linear term of incubation; Table 2). Although catalase treatment did not affect the shape of the incubation curve ($P>0.05$ for the effect of catalase treatment on the cubic and quadratic term of incubation; Table 2), BP M_{pos} values of catalase-treated sperm showed lower values after 24h of incubation in comparison to the control group ($P<0.05$ for the effect of catalase treatment on the linear term of incubation; Table 2). There were no differences in BP M_{pos} values between sperm frozen with different catalase concentrations at all distinct time points after thawing ($P>0.05$, Table 1).

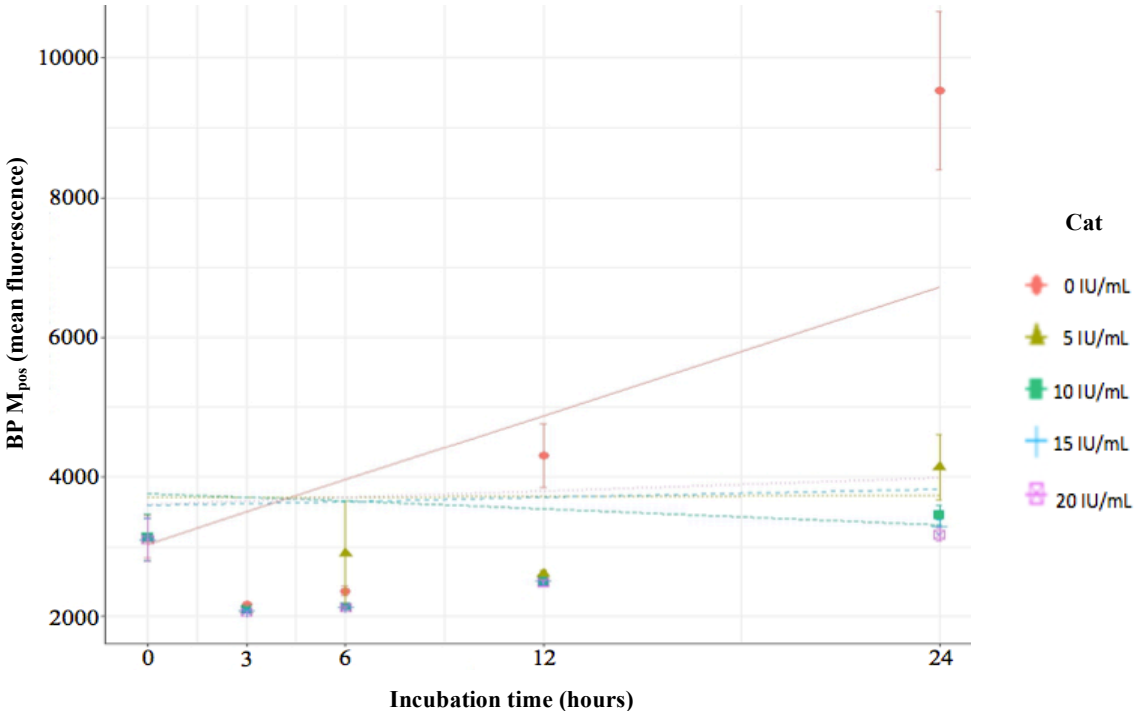

Figure 8: Mean values (±SEM) and model-predicted time curves of bodipy fluorescence in cryopreserved bovine sperm with a high mitochondrial membrane potential (BP M_{pos}) diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

Catalase-treated sperm showed a lower increase of %DFI values in comparison to the control group at 24h ($P<0.05$ for the effect of catalase on the linear term of incubation for all doses; Table 2). In the same time, as shown in Figure 9, catalase treatment had an effect on the shape of incubation curve ($P<0.05$ for the effect of catalase treatment on the quadratic and cubic term of incubation; Table 2), which implied a smoother increase of %DFI in the catalase-treated sperm compared to the control. At 12 h and 24 h after thawing the %DFI values were lower in sperm frozen with all different concentrations of catalase than in sperm frozen without catalase. In addition, at 24 h after thawing sperm frozen with concentrations of 10, 15 and 20 IU catalase/mL showed lower %DFI values than sperm frozen with 5 IU catalase/mL ($P<0.05$, Table 1).

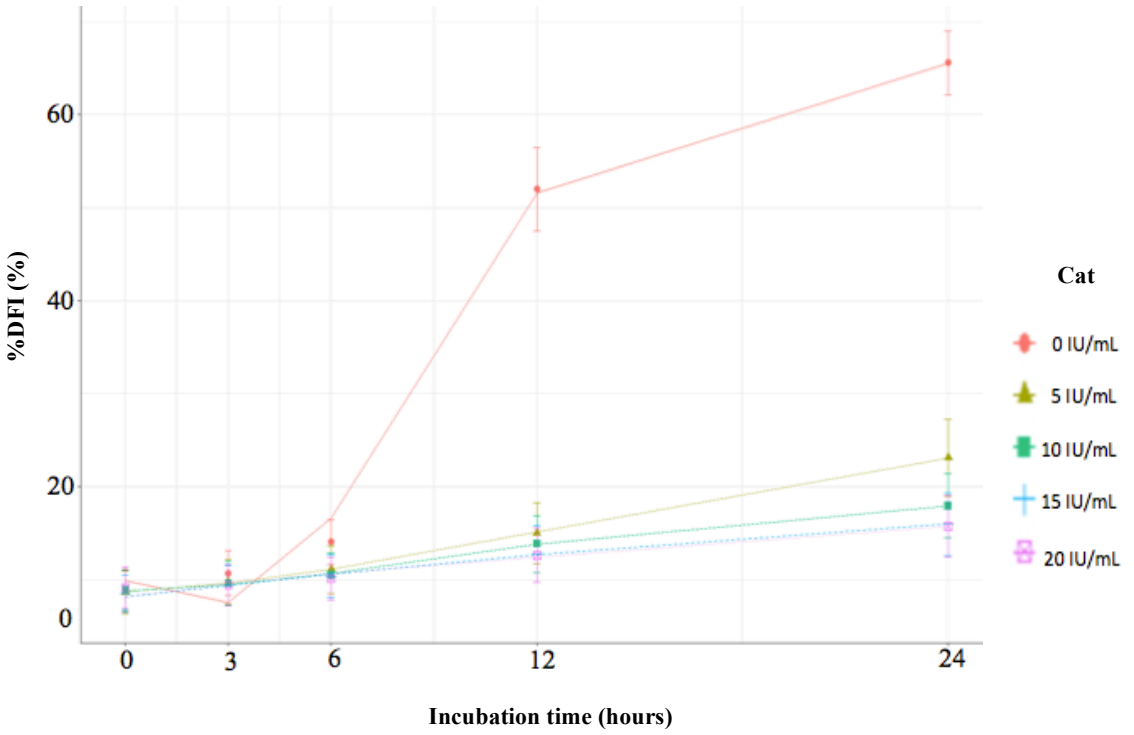

Figure 9: Mean values (±SEM) and model-predicted time curves of cryopreserved bovine sperm with a high DNA fragmentation index (% DFI) diluted with Triladyl® extender without (red lines) and with the addition of different catalase (Cat) concentrations (dark green 5 IU/mL, green 10 IU/mL, blue 15 IU/mL and purple 20 IU/mL lines). In each of 7 bulls, 2 ejaculates were examined.

As the best sperm quality was obtained with extender containing 20 IU catalase/mL, sperm cryopreserved with this Cat-concentration were used for *in vitro* fertilization. The effects of control sperm and sperm frozen with 20 IU catalase/mL were compared. There were no differences in the cleavage rate, blastocyst rate and hatching rate between the sperm frozen without and with the addition of 20 IU catalase/mL (P>0.05, Table 3).

Table 3: Cleavage of cells (Cleavage), development of blastocysts (Blastocysts) and hatching of embryos (Hatching) after *in vitro* fertilization of oocytes with cryopreserved sperm frozen in extender without (Control) and with the addition of 20 IU/mL catalase (Catalase).

Sperm	Oocyte	Cleavage		Blastocysts		Hatching	
	Number	Number	Rate	Number	Rate	Number	Rate
Control	110	97	88%	78	71%	59	67%
Catalase	101	90	89%	67	67%	57	85%

4. DISCUSSION

The results of the study show that the addition of catalase to the semen extender before freezing had a positive effect on sperm quality. However, this effect could not be seen immediately, but only after several hours of incubation after thawing. A number of other studies demonstrated also a positive effect on the quality of cryopreserved bovine sperm, but in contrast to our study already immediately after thawing [4, 8, 49]. On possible reason for these differences could be that compared to our experiments in the trials cited above higher concentrations of catalase have been added to the extender (> 100 IU/mL). However, before our main trials we performed some preliminary experiments with catalase concentrations between 20 IU and 200 IU before our main trials and could not find any significant differences in the effects of such a high range of catalase concentrations on sperm quality (data are not shown). Another reason for the contradictory results compared to the literature might be the use of different extenders for cryopreservation of sperm. The extenders contain different amounts of various antioxidants and show therefore differences in their antioxidative capacity [4, 8, 37 49], As most of the extenders like in our study are commercially produced, their exact composition is not known [50, 58, 64]. It could be that the extender used in our study contained more antioxidants than the extenders used in the other studies and therefore effects of the antioxidant catalase could not be noticed immediately after thawing, but after several hours of incubation as soon as oxidative stress increased after the consumption of most the antioxidants in the extender. Another reason for the discrepancies in the results of our trial and those of other studies could be the freezing process. In contrast to the catalase studies described in the literatures [20, 49, 55], we equilibrated the sperm much longer. It is well known that the type of the cryopreservation process could affect the oxidative stress [1, 56].

The addition of catalase affected all investigated sperm characteristics, motility, plasma membrane and acrosome integrity, metabolism (mitochondrial membrane potential, esterase activity, calcium level), DNA integrity, lipid peroxidation and synthesis of ROS. While higher values for motility, plasma membrane and acrosome integrity and metabolism of sperm were noticed, DNA integrity lipid peroxidation and ROS synthesis were lower after the addition of

catalase to the extender. These findings are in accordance with studies in which supplementation of exogenous catalase improved motility [4], plasma membrane integrity [37, 49], mitochondrial potential [44], DNA integrity [4] and lowered ROS [55] and LPO [51], values. Since catalase as an antioxidant has various effects on different cell characteristics the multiple sperm parameters are not surprising [18].

The most sensitive parameter for the investigation of the positive effect of catalase on sperm quality in our study was the CASA parameter RMS. Using this parameter differences between sperm samples with and without the addition of catalase could be observed already after 3 h of incubation of frozen-thawed sperm, while all other sperm parameters did not differ depending on the addition of catalase before 12 h after thawing. This finding seems to be a bit surprising, because the flow cytometric sperm parameter $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ includes the combined evaluation of five different sperm characteristics. However, it should be noticed that motility depends on all these sperm characteristics determined by the multicolour assay described above [52]. In addition, the described multicolour assay differentiates only between sperm showing high and low esterase activities, mitochondrial membrane potentials and calcium concentrations as well as sperm with intact and defect plasma membrane integrities, while the CASA system differentiated not only motile from immotile sperm, but also rapid from slow motile sperm. This fact could be a reason for the higher sensitivity of the parameter RMS compared to the flow cytometric parameters in the investigation of the effect of exogenously catalase in cryopreserved bovine sperm.

The addition of catalase showed tremendous effects on the amounts of LPO, ROS and DNA damages in sperm. While in sperm frozen with extender without catalase distinct changes in these sperm parameters were noticed, the same sperm characteristics showed only slight increases during the 24 h incubation period after thawing. It is well known that mammalian sperm membranes contain high amounts of polyunsaturated fatty acids and thus are very sensitive to oxidative stress [1]. Increased ROS and LPO levels following freezing and thawing of sperm have been shown in numerous studies [12, 14, 39]. Da Silva et al (2010) demonstrated a rise of H_2O_2 generation in sperm and could inhibit this increase by addition of catalase to the semen extender [25]. In another study a rise in LPO levels of bovine sperm could be inhibited by the addition of catalase to the freezing extender [51]. It has also been shown that the processes of freezing and thawing induce an increase of DNA damage in different species (bovine [64], ram [50], equine [10], canine [35], human [48]). Gürler et al. (2016) noticed that the DNA damage is related to the rise in H_2O_2 concentrations during incubation of frozen-thawed bovine sperm [36] and Ali et al. (2017) showed that the addition of catalase to the freezing extender enhanced DNA integrity in buffalo sperm [4]. Therefore, it can be suspected that the positive effects of catalase on DNA integrity are based on the inhibition of H_2O_2 synthesis and thus by the decrease of oxidative stress.
Comparing the effects of different catalase concentrations, it became obvious that the addition of 20 IU of catalase/mL was most effective in improving sperm quality in cryopreserved sperm. After 3 h of incubation the RMS values were higher in frozen-thawed sperm cryopreserved with 15 and 20 IU compared to 5 IU catalase/mL. Furthermore, plasma membrane and acrosome integrity as well as $C_{pos}M_{pos}F_{neg}PI_{neg}PNA_{neg}$ values and DNA integrity were higher in sperm frozen with 10, 15 and 20 IU of catalase compared to sperm cryopreserved with 5 IU catalase/mL after 24 h of incubation. In addition, 10 and 20 IU catalase, but not 15 IU catalase lowered the amount of ROS synthesis compared to sperm frozen with 5 IU catalase/mL in the extender at 24 h of incubation after thawing. As our study was the first one investigating dose-effect relationships between catalase and sperm characteristics in cryopreserved bovine sperm, we are not able to compare our findings with those of other groups.

In contrast to the observed improvement in sperm characteristic, we did not detect any differences in the rates of cleavage and development and hatching of blastocysts following *in vitro* fertilization with sperm frozen without and with the addition of 20 IU catalase/mL. Similar results were obtained in comparable studies (bulls [3], goats [58], Iberian Ibex [45]). This finding seems also to be surprising, because it has been demonstrated that the amount of DNA damage are negatively related with fertilizing potential of sperm either *in vivo* [60] or *in vitro* [7, 61]. However, to explain this astonishing result it should be emphasized that compared to sperm cryopreserved without catalase the addition of this antioxidant did not improve DNA integrity before 12 h after thawing of frozen sperm. *In vitro* oocytes are fertilized by bovine sperm within the first 4 h after thawing [19]. At this time point sperm frozen without and with catalase showed the same percentage of DNA intact sperm in our study. Furthermore, it should be noticed that a Percoll centrifugation was performed immediately after thawing to separate viable sperm with a normal morphology from dead sperm and sperm with an abnormal morphology. It has been shown in several studies that the Percoll centrifugation also separates sperm with a high DNA integrity from sperm with DNA damages. This could be another reason, why the addition of catalase showed no positive on embryo development *in vitro*. As *in vitro* fertilization occurs not immediately, but several hours after thawing of sperm and the sperm are not selected by a Percoll centrifugation it cannot be concluded from the *in vitro* studies that the addition of catalase to the extender before freezing shows no effect on *in vivo* fertility. Therefore, such studies should be performed in the future.

In conclusion, the results of our study show that the addition of catalase to a TRIS-egg yolk extender has positive effects on a number of sperm characteristics, but only several hours after thawing. There are no positive effects of the addition of catalase on *in vitro* fertility, but the impact on *in vivo* fertility has to be clarified in future studies.

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